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A CHEMICAL EXAMINATION OF VARIOUS TUBERCLE BACILLI.

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The preliminary work of Hammerschlag [1a] upon the substances contained in the bodies of tubercle bacilli which could be extracted with ether and alcohol, and the probable composition of the extracts so obtained, offered considerable material for speculation. Nothing further was done in this line, however, until we [2] reported investigations which we had made confirming the work of Hammerschlag indicating the presence of a large percentage of ether and alcohol soluble material in tubercle bacilli. By a chemical examination of these extracts we were also able to determine the presence of volatile fatty acids, together with other acids of the fatty series of which only the melting points were determined.

More recently Klebs [3] reported the presence of considerable fatty material in tubercle bacilli. Weyl [4] found that the fatty extract of tubercle bacilli was possessed of the same "acid fast" property that is exhibited by the bacilli themselves. Ruppel [5] claims to have isolated three different fatty substances from the tubercle bacilli. Several years after we had published our preliminary work Aronson [6] reported a chemical examination which he had made of the bodies of tubercle bacilli. In this article he claims that a very large proportion of the ether and alcohol soluble material consists of free fatty acids. This statement, however, is not entirely confirmed by our own work. Levene [7] has reported analyses of human tubercle bacilli which were grown upon the ordinary glycerine bouillon and upon a similar medium containing mannite. By these analyses he found a considerable variation in both the percentage of extractive material and in the percentage of ash obtained after burning. In a recent article Kresling [8] reviews the work of various authors relating to the chemical constitution of tubercle bacilli of human origin, and also reports the results of his own analyses. The order in which the several solvents were used was varied considerably. The percentage obtained, however, was in all cases approximately the same. The bacilli had been collected during a number of years and had been cultivated upon the ordinary glycerine bouillon containing sodium chloride and peptone. Kresling found that the chloroform extract, chloroform being the first extractive used, contained about 14 per cent of free fatty acid.

[a] The numbers refer to the bibliography at the end of the article.

We have extended our studies in the examination of the bodies of tubercle bacilli to the following: (1) An attenuated bacillus of human origin, obtained originally through the courtesy of Dr. E. L. Trudeau, which had been derived from a man and passed through a guinea pig. This had been grown upon glycerinized bouillon for about one hundred and sixty generations. Although originally virulent for small animals, it had entirely lost its pathogenic properties for guinea pigs. (2) A virulent bacillus of human origin, obtained also from man, which had subsequently been grown for fifty generations upon glycerinized bouillon. This organism still retained its virulence and was capable of causing the death of guinea pigs in five or six weeks after subcutaneous injection of a small amount. (3) A virulent bovine bacillus obtained originally from Dr. Theobald Smith, which was still virulent for small animals as well as for cattle. (4) A swine bacillus, obtained also through the kindness of Dr. Theobald Smith, virulent for small animals. (5) A tuberculosis bacillus from a horse, obtained from Doctor Ravenel, of Philadelphia, and regarded by him as originally of bovine origin, as the conditions indicated that the horse had contracted the disease from cattle. (6) An avian bacillus, the original culture of which was also obtained from Doctor Ravenel. The bovine, swine, horse, and avian bacilli had also been grown upon glycerinized bouillon for several years, without passage through an animal. In order to obtain a quantity of material sufficiently large to permit of the chemical analyses recorded below, it was necessary to grow these various organisms in quantity; and in order to make the analyses comparable, a uniform medium was used. Its preparation and composition can be seen from the following record, taken from the laboratory books in which records are kept of each lot of culture media prepared: Chopped meat 1 part, distilled water 2 parts, heated at 45° to 58° C. for three hours, strained, boiled, filtered, 1 per cent peptone and one-half of 1 per cent acid potassium phosphate added. Neutralized with sodium hydrate; boiled one hour, 7 per cent glycerine added, filtered; acidity about 10 c. c. N/10 sodium hydrate required to neutralize 100 c. c. beef broth, phenolphthalein being used as an indicator.

The cultures of the several tubercle bacilli made upon the medium above described were from two to four months old, and had been grown at a temperature of 37° to 38° C. The whole cultures were poured into perfectly clean, sterile flasks and heated for a few minutes to the boiling point. The bacilli were then allowed to settle and first washed by decantation (with hot water), then transferred to a folded filter and washed again with hot water so long as a reaction for phosphates was noted, when the filtrate was tested with silver nitrate. This same process was adopted for all the different cultures, the greatest care being used to avoid contamination with any foreign matter. The moist germs were carefully removed from the filter paper by means of a spatula and dried over sulphuric acid. They

were then broken up into pieces about the size of very small bird shot and dried to a constant weight at 60° C. in an oven with a vacuum of 26 inches. These dried bacilli served as the starting point for the extractions. They were extracted first with hot ether, then with hot alcohol, and last of all with hot chloroform, Knorr's extraction apparatus being used. In each case the extraction was continued as long as any material was dissolved, and the extracted substance was again dried to a constant weight before proceeding to use the next solvent. The time occupied in this operation with the different solvents was from four to five days each. The quantity of material extracted was determined by loss of weight, the germs used being first weighed in the tube and again after the different extractions had been completed. The error resulting from manipulation, therefore, was the minimum. Table I shows the results of duplicate determinations of the ether, alcohol, and chloroform extracts of the six varieties of tubercle bacilli examined.

TABLE I.—*Ether, alcohol, and chloroform extracts of tubercle bacilli.*

	Bovine bacilli.			Swine bacilli.			Horse bacilli.		
	1.	2.	Average.	1.	2.	Average.	1.	2.	Average.
	Per ct.	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*
Ether extract	17.74	17.66	17.70	13.69	11.43	12.56	22.90	23.87	23.38
Alcohol extract	.....	8.13	8.13	.....	7.83	7.83	8.18	8.18	8.18
Chloroform extract	.....	.49	.49	.....	.20	.20	.29	.12	.20
Total	.....	26.28	26.32	.....	19.46	20.59	31.37	32.17	31.76

	Avian bacilli.			Attenuated human bacilli.			Virulent human bacilli.		
	1.	2.	Average.	1.	2.	Average.	1.	2.	Average.
	Per ct.	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*
Ether extract	17.40	17.32	17.36	28.86	28.59	28.72	20.40	20.22	20.31
Alcohol extract	13.15	13.39	13.27	7.22	7.49	7.36	7.21	7.23	7.22
Chloroform extract	.04	.....	.02	1.33	(1.33)	1.33	.48	(.48)	.48
Total	30.59	30.71	30.65	37.41	37.41	37.41	28.09	27.93	28.03

It will be noted that by far the highest percentage of ether extract is obtained from the attenuated human bacilli, and from the others in the following order: Horse bacilli, virulent human bacilli, bovine bacilli, avian bacilli, and swine bacilli. The percentage of ether extract obtained from the avian and bovine bacilli was about equal in amount. When we consider the alcohol extract the order is different. The highest percentage of alconol extract is found in the avian bacilli, and the others in the following order: Horse, bovine, swine, and human bacilli, there being but little difference between the quantity of alcohol extract in the two varieties of human bacilli. The chloroform extract was hardly worth consideration, except in the case of the attenuated

human bacilli and in the case of the virulent human bacilli and the bovine bacilli. In these latter the percentage of chloroform extract appeared to be about the same. In the consideration of the totals of these various extracts we find the following order: The highest percentage in the attenuated human, the horse, avian, virulent human, bovine, and swine, following in the order named, as can be seen in Table I. This high percentage of total extractive matter in the attenuated human bacilli agrees fairly well with the percentage noted in our original work upon the human bacilli already referred to and reported in 1895.

The amount of material at our disposal was so small that it was found to be impracticable to undertake an extensive examination of the ether and alcoholic extracts of the various bacilli. We have, however, been able to determine the percentage of free fatty acid in these extracts. The results of these determinations are recorded in Table II. The free fatty acid was calculated from the acid value, which was determined by titration with N/10 sodium hydrate. The free acids were calculated in all instances as oleic, although we do not feel at all certain that the acid value is due to the presence of oleic acid. An examination of Table II will show that the highest acid value was found in the extracts from the virulent human, and from the others in the following order: Swine, attenuated human, avian, bovine, and horse tubercle bacilli.

TABLE II.—*Acid value of ether and alcohol extracts of tubercle bacilli.*

	Acid value.		Total acid value calculated on the sum of the ether and alcohol extract.	Free acids in alcohol and ether extracts.	
	Ether extract.	Alcohol extract.		Ether extract.	Alcohol extract.
				Per cent.	*Per cent.*
Bovine bacilli	9.43	20.40	12.90	4.74	10.25
Swine bacilli	8.02	23.45	13.97	4.03	11.78
Horse bacilli	9.36	18.47	11.46	4.70	9.28
Avian bacilli	13.00	13.11	13.04	6.53	6.59
Human bacilli (attenuated)	12.77	14.57	13.13	6.41	7.32
Human bacilli (virulent)	14.02	16.45	14.63	7.04	8.26

	Percentage of free acids calculated on the ether and alcohol extracts combined.	Free acids calculated for whole substances.		Total per centage free acids in bacilli.
		Ether extract.	Alcohol extract.	
	Per cent.	*Per cent.*	*Per cent.*	*Per cent.*
Bovine bacilli	6.48	0.83	0.83	1.66
Swine bacilli	7.02	.46	.92	1.38
Horse bacilli	5.76	1.09	.75	1.84
Avian bacilli	6.55	1.13	.87	2.00
Human bacilli (attenuated)	6.60	1.84	.53	2.37
Human bacilli (virulent)	7.35	1.42	.59	2.01

NOTE.—The free acids were calculated from the acid value and were considered as oleic acid.

Following our earlier work, we have had ash determinations of the various bacilli made, and in addition a determination of the phosphoric acid present in the ash. (These analyses were kindly made by Mr. James A. Emery, of this laboratory.) As the bacilli used for these determinations had all been grown upon the same sort of medium, washed in the same way with approximately the same amount of water, it is fair to presume that the percentage of ash represents approximately, if not absolutely, the ash which would result from a destruction of the organic matter of these bacteria. Sulphuric acid and chlorides were not found, and the high percentage of phosphoric acid seems to be a common characteristic of tubercle bacilli.

TABLE III.—*Ash and phosphorus in tubercle bacilli.*

	Moisture.		Ash.		P_2O_5 in dry bacilli.		P_2O_5 in ash.	
	1.	2.	1.	2.	1.	2.	1.	2.
	Per ct.	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*
Bovine bacilli	2.42	2.48	2.66	2.67	1.56	1.55	58.54	58.04
Swine bacilli	2.26	2.06	2.37	2.31	1.30	1.31	55.	56.48
Horse bacilli	2.27	2.42	3.63	3.55	2.07	2.02	55.68	55.40
Avian bacilli	2.40	.....	3.96	3.94	2.22	2.19	55.98	55.63
Human bacilli (attenuated)	2.67	2.58	2.44	2.31	1.79	1.71	73.49	74.38
Human bacilli (virulent)	3.91	3.70	3.94	3.92	2.50	2.38	63.47	60.90

In our earlier article upon the mineral constituents of the tubercle bacilli, published in 1898, the percentage of phosphoric pentoxid was found to be a little over 55 in the human germ. As will be noted from Table III, the percentage of phosphoric pentoxid in these human germs was found to be over 60 per cent in the virulent and over 70 per cent in the attenuated. In comparing these figures with the earlier results it should be remembered that the germs used for obtaining the earlier data were grown upon the ordinary glycerine bouillon to which no phosphates had been added, while all of the examinations here recorded dealt with bacilli which were grown upon a medium rich in phosphates. The amount of material available for the phosphoric pentoxid determinations was very small, so that there may be some error due to manipulation, but, allowing for these facts, it is very evident that all of these tubercle bacilli are voracious consumers of phosphoric oxide, in which property they correspond to a great many other plants.

Until we have completed the determination of the exact character of the ether, alcohol, and chloroform extracts obtained from these various bacilli, which are in progress at present, much speculation in regard to the relation of the composition of any one of these organisms to its character and virulence is not warranted. It is interesting to note that the percentage of alcohol extract obtained from the avian bacilli is very much greater than that obtained from any of the other

organisms which were examined, while the percentage of chloroform extract in the bovine bacilli and virulent human bacilli is almost exactly the same, there being but little chloroform extract obtained from the horse, swine, and avian bacilli, while a considerably larger amount is secured from the attenuated human bacillus. The variation in the amount of ether extract is also noticeable, the attenuated human organism showing the largest percentage.

The results which we have obtained certainly indicate that there may be a wide variation in the chemical composition as well as in the morphology and pathogenic power of tubercle bacilli. The results which we have presented here are not sufficient, however, to enable us to draw any definite conclusions in regard to the constant chemical differences, if any really exist, between the several varieties of tubercle bacilli. It will be seen that there may be as great a difference between an attenuated and a virulent human tubercle bacillus as there is between an attenuated human bacillus and a bovine organism, and there seems to be no reason for supposing that there may not be bovine bacilli which will correspond entirely with certain human bacilli in so far as their chemical constitution is concerned. In fact, these results considered alone show only that there may be a wide variation in the chemical composition of tubercle bacilli. They do not indicate that there is necessarily any specific relation between their chemical composition and their virulence. The horse tubercle bacilli, although distinctly more virulent than the most virulent human bacilli which we have analyzed, contain a higher percentage of ether soluble material, and are chemically more closely related to the very attenuated human bacillus than the virulent human bacillus above mentioned. Before drawing any conclusions whatever, therefore, we must wait for analyses of other cultures of tubercle bacilli from various sources, after which it may be that some definite conclusions in regard to the relation between the virulence and the chemical composition may be formed.

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